IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants:	Yukoh HIEI et al		
Serial No.:	10/089,695	Group:	1638
Filed:	May 21,2002	Examiner:	Worley
For:	METHOD FOR PRODUCING EFFICIENTY OF GENE TRANSFER INTO PLANT CELLS		

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Office Action dated April 25, 2008, in which claims 1, 12, 14, 15, 17 and 19-21 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

EXPERIMENTS

Efficiency of *Agrobacterium*-mediated gene transfer in rice immature embryos pre-treated with various temperatures (25, 37 or 52 °C) and the durations.

Materials and Methods

(1) *Agrobacterium* Strain and Plasmid

As the *Agro*bacterium and its vector, LBA4404(pTOK233) (Hiei et al. 1994) was

used. The T-DNA region of pTOK233 has a hygromycin-resistant gene (*hpt*) regulated by the 35S promoter of CaMV and a GUS gene regulated by the 35S promoter of CaMV and having the first intron of the catalase gene of castor-oil plant.

(2) Sample Varieties and Tissue

As the sample varieties, IR64, which is the variety of Indica rice, and Yukihikari, which is the variety of Japonica rice, were used. As the sample tissue, immature embryo was used. The preparation method of the tissue is the same as that described in the specification of the present patent application.

(3) Heat Treatment

Rice immature embryos were placed in a 1.5 ml centrifugal tube containing 1 ml of sterilized water. The tubes containing immature embryos were incubated in a water bath at various temperatures (25, 37 and 52 °C) before infection with *A. tumefaciens* strain. The duration of the treatment was 3, 6, 12 or 24 hr at 25 or 37 °C and 1, 3, 5 or 7 min at 52 °C for the immature embryos of IR64. For the immature embryos of Yukihikari the duration of the treatment was 10 or 20 hr at 25 or 37 °C. After the heat-treatment, the tubes were cooled in on ice for 30 sec. In addition to the experimental plots, an experimental plot with no heat-treatment (room temperature, around 20 °C) was added. The immature embryos of no-heated control were inoculated with *Agrobacterium* immediately after the isolation.

(4) Infection of *Agrobacterium* and Co-culturing

The method of infection of the immature embryos with *Agrobacterium*, the method of co-culturing and the method of GUS assay of the immature embryos after the co-culturing were the same as described in specification of the present patent application. The co-culturing was carried out for 7 days. In the present test, the GUS expression levels in the grown immature embryos were expressed in values as GUS Activity Index as follows: Each of the grown immature embryos was then visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score

was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for 75% and 100%. The average of the scores in an experimental plot was recorded as the GUS Activity Index. The percentage (the GUS Activity Index) was then divided by the percentage for the untreated control plot to give a relative value.

Results

An enhancement of GUS expression in the grown immature embryos by the pre-treatment with heat was observed after the co-cultivation. The general tendency was that the higher the temperature of the treatment for the immature embryos of IR64, the shorter was the time for the level of GUS expression to reach the peaks, which were detected after 120 min of treatment at 37 °C (Figure 1) and 3 min at 52 °C (Figure 2) in the tested range. The height of the peaks differed between the treatments at 37 and 52 °C (Figure 1 and 2). It seems the effect of the heat-treatment at 37 °C is somewhat lower than that of higher temperature. However, an effect by the pre-treatment at 37 °C to improve gene introduction efficiency was observed even if the pre-treatment was carried out for the long time like 12 hours or 24 hours to immature embryos (Figure 1). The same effect for gene introduction was also observed in the immature embryo of Japonica rice Yukhikari pre-treated at 37 °C for 10 or 20 hours (Figure 3).

Cited Reference

Hiei Y, Ohta S, Komari T & Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282

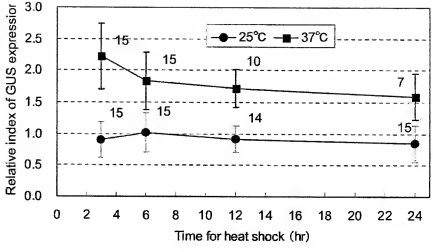

Figure 1. Relative GUS expression in immature embryos of rice variety IR64 pre-treated with heat at 25 °C or 37 °C.

Immature embryos of IR64 were treated with heat at 25 °C or 37 °C for defined lengths of time. The embryos were then inoculated with *A. tumefaciens* LBA4404(pTOK233). GUS in the grown immature embryos after the completion of co-cultivation was assayed histochemically with X-gluc. The GUS expression relative to that in no-heat treated control is plotted with the range of standard error. The number of investigated embryos was recorded in the graph.

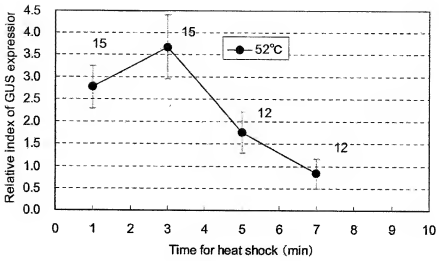

Figure 2. Relative GUS expression in immature embryos of rice variety IR64 pre-treated with heat at 52 °C.

Immature embryos of IR64 were treated with heat at 52 °C for defined lengths of time. The embryos were then inoculated with *A. tumefaciens* LBA4404(pTOK233). GUS in the grown immature embryos after the completion of co-cultivation was assayed histochemically with X-gluc. The GUS expression relative to that in no-heat treated control is plotted with the range of standard error. The number of investigated embryos was recorded in the graph.

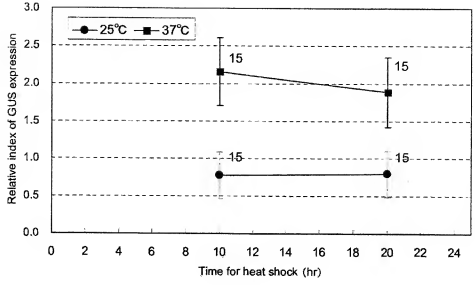

Figure 3. Relative GUS expression in immature embryos of rice variety Yukihikari pre-treated with heat at 25 °C or 37 °C.

Immature embryos of Yukihikari were treated with heat at at 25 °C or 37 °C for defined lengths of time. The embryos were then inoculated with *A. tumefaciens* LBA4404(pTOK233). GUS in the grown immature embryos after the completion of co-cultivation was assayed histochemically with X-gluc. The GUS expression relative to that in no-heat treated control is plotted with the range of standard error. The number of investigated embryos was recorded in the graph.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of

the application or any patent issuing thereon.

This 12 day of June, 2009

Yukoh HIEI